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The interaction of specific T-cell help and non-specific B-cell growth factors in the production of anti-tetanus antibody by human B cells grown in serum-free microcultures

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Summary. Apart from a brief period after *in-vivo* immunization, only a minority of human donors provide peripheral lymphocytes that synthesize specific antibody on stimulation with tetanus toxoid *in vitro*. A 20 μl hanging drop microculture technique using serum-free medium has been adapted to analyse the conditions under which B cells mature into antibody-secreting cells. Multiple permutations of antigen dose, cell concentration and T:B cell ratios have been examined. The results indicate that *in-vitro* failure of antigen response by the majority of donors is not due simply to an inappropriate choice of culture conditions. The addition to antigen-stimulated cultures of a lectin-free conditioned medium derived from pokeweed mitogen-stimulated peripheral lymphocytes, enables B cells from the majority of donors to produce high titres of specific antibody, in a T-dependent manner, for up to 24 months after immunization. The observed failure of prolonged antigen responsiveness *in vitro* thus appears to represent a failure to expand a population of antigen-specific B cells, rather than indicating an absence of such clones.

Abbreviations: Ab, antibody; BSA, bovine serum albumin; CM, lectin-free conditioned medium; FACS-2, fluorescence-activated cell sorter (Becton-Dickinson); FCS, foetal calf serum; Ig, Immunoglobulin; IMDM, IMDM(U), IMDM(TAS), Iscove's modified Dulbecco's Medium, unsupplemented (U) or supplemented with transferrin, albumin, soybean lipid (TAS); n-SRBC, neuraminidase treated sheep red cells; PBM, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PWM, pokeweed mitogen; TRF, T-cell replacing factor; TT, tetanus toxoid.

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INTRODUCTION

Only 15% of individuals provide peripheral blood lymphocytes able to respond to antigen in culture, for more than a brief period after immunization *in vivo*: lymphocytes from most donors fail to produce specific antibody after antigen stimulation *in vitro*, even when the cells have been obtained just a few weeks after immunization (Brenner, Bright & Munro, 1981; Mitchell *et al.*, 1982). Repeated vaccination of non-responders may not be possible, and can provide a little information about the long-term maturation of the specific immune response following a single exposure to antigen.

The explanation of this phenomenon has not been clear. *In-vitro* unresponsiveness cannot be overcome by using large-scale cultures of lymphocytes from these immune non-responders (Brenner *et al.*, 1981), so it is unlikely simply to be a consequence of a low frequency of circulating antigen-specific lymphocytes.

We have examined two other explanations. First, unresponsiveness may be an indication that as the immune response matures after vaccination, there is an associated reduction in the combination of cell concentrations and T and B cell ratios that are effective for *in-vitro* antibody synthesis. Conventional culture techniques need substantial numbers of lymphocytes, reducing the range of culture conditions that can be studied (Mudawwar, Yunis & Geha, 1978; Callard, 1979; Brenner & Munro, 1981a), so the precise combination necessary for a detectable response might be missed. We have therefore used a microculture system, in which cells are grown in 20 μl hanging drops. This system allows several culture variables to be examined simultaneously, and has permitted exploration of the influence of culture conditions on long-term responsiveness.

An alternative explanation for unresponsiveness is that antigen-reactive T and B cells in culture lack additional non-specific proliferative and differentiation factors that may be necessary to amplify these initial, specific, cell interactions (Schimpl & Wecker, 1975; Farrar *et al.*, 1982; Swain *et al.*, 1982). We have examined this possibility by adding lectin-free growth and differentiation factors derived from mitogen stimulated lymphocytes. Serum-free medium is used in the microculture system, permitting clearer interpretation of the effects of adding exogenous factors: allogeneic or xenogeneic sera are often mitogenic, and sometimes contain growth factors themselves.

The experiments in this paper show that optimal antibody production by the cells of *in-vitro* responders occurs over a narrow range of culture conditions. This range can be increased if the appropriate growth factors are added to the antigen stimulated cultures. Under these circumstances, many donors whose lymphocytes failed to respond *in vitro* to antigen alone, secrete significant quantities of specific antibody, even when their cells are obtained up to 24 months after immunization.

MATERIALS AND METHODS

Media

The culture medium used was essentially as devised by Iscove and Melchers, but contained different quantities of transferrin, delipidated albumin and soybean lipid. Iscove's modified Dulbecco's medium (IMDM) was prepared from powder (1 litre pack, Gibco, Cat. No. 430-2200, Batch No. E096550), which was dissolved in water for injection (Antigen Ltd) in the dark and sodium bicarbonate (3·024 g Analar) added. The medium was sterilized by filtration through 0·22 μm millipore filters (Millex GS) and stored at 4°. The solution was 280 mosmolar and its pH when equilibrated with CO_2 10% in air at 37° was 7·2.

The additives transferrin (T), albumin (A) and soybean lipid (S) were prepared as previously described (Iscove & Melchers, 1978; Iscove, Guilbert & Weyman, 1980) and after filter sterilization were stored at 4°. One hundred milligrams of human transferrin (Sigma, T-2252, lot 120f-0381) was dissolved in 1·06 ml of IMDM containing penicillin/streptomycin (100 IU/ml) and 50 μl of $FeCl_3$ (at 4 mg/ml in 0·1 mM HCl) was added. Purified bovine serum albumin (10 g; BSA, Behring, Lot 225041B) was delipidated (Iscove & Melchers, 1978) using activated charcoal (Norit OL), deionized with Amberlite (MB-1) and concentrated over an Amicon membrane (YM10) to 5% w/v and the pH adjusted to 7·0. Soybean lipid (Natterman PH75), 20 mg in 20 ml unsupplemented Iscove's medium—IMDM(U)—at pH 6·8 in the presence of 1% delipidated BSA, was sonicated in a 25 ml beaker with internal dimensions of 30 mm wide, 45 mm deep, for 5 min on ice at maximum amplitude using a 1-cm diameter probe (MSE soniprep).

Preparation of culture medium

Immediately before use, penicillin (100 IU/ml), streptomycin (100 IU/ml) and L-glutamine (2 mM/ml) were added to the medium now designated IMDM(U). For the completely supplemented medium IMDM(TAS), the following were added to 20 ml of IMDM(U): transferrin (90 mg/ml, 1/3 saturated with iron, 5 μl), delipidated BSA (5%, 400 μl) and sonicated soybean lipid (400 μl). The final concentration of transferrin was 22·5 μg/ml, of BSA 1 mg/ml and of lipid 20 μg/ml.

Cells and cultures

Mononuclear cells were obtained from the peripheral blood of exercised normal human volunteers who had been immunized with Adsorbed Tetanus Toxoid (Wellcome) 3–24 months previously. The blood was defibrinated, centrifuged at 900 *g* for 5 min and the serum removed and kept on ice. The blood was then diluted with Leibovitz L-15 Medium (Flow), layered onto Ficoll-paque (Pharmacia) and centrifuged at 650 *g* for 35 min. The cells harvested from the interface were washed twice and adjusted to 5×10^6/ml in L-15, to which 10% heat-inactivated (HI) pooled human AB serum had been added. Each 5 ml aliquot was incubated at 37° for 90 min in a 5-cm diameter petri-dish (Nunc). The non-adherent cells were removed gently and washed twice in L-15 and adjusted to 3×10^6/ml in L-15 containing 10% heat-inactivated foetal calf serum (Gibco, L285601S; FCS). T and non-T cells were obtained as follows: to every 3 ml of this cell suspension, 1 ml of neuraminidase-treated sheep red blood cells (n-SRBC) were added. To prepare n-SRBC 1 ml of washed and packed SRBC (Flow) and 0·3 ml of Neuraminidase (1 IU/ml; Behringwerke) in 10 ml of phosphate-buffered saline (PBS) were incubated for 35 min at 37° and washed three times in PBS.

The cell suspension with n-SRBC was centrifuged at 160 ***g*** for 5 min and left at room temperature for 1 hr. The pellet was gently resuspended and layered onto Ficoll-paque and centrifuged for 35 min at 650 ***g***. The cells at the interface were removed, washed three times, resuspended in IMDM(TAS) and used as the non-T preparation.

The cell pellet containing the rosettes (T cells) was mixed gently with L-15 (1 ml). Donor serum (3 ml) was added and the whole incubated at 37° for 10 min. After the red cell complement lysis, the T cells were washed and pooled. When appropriate the T cells were irradiated (2000 rads), from a Cobalt 60 source at 8 rads/sec). Aliquots (10 μl) of twice the final required concentration of non-T cells were dispensed into Terasaki plates using a repeating Hamilton syringe. Similar aliquots of T cells were added. Finally, the stimulant was added in a 1 μl aliquot, to give a final well volume of 21 μl. The cells were cultured by inverting the Terasaki plates and placing them in humidified boxes in a 5% CO_2 incubator as previously described (Farrant *et al.*, 1980).

Antigen

Tetanus Toxoid (TT) at 2500 IU/ml was kindly supplied by the Wellcome Foundation. It was dialysed three times against PBS and passed through a 0·22 μm filter.

Mitogen

PWM (Sigma) was obtained in freeze-dried form and after reconstitution, stored at −70°.

Experimental design of cultures

The design of wells in the plates was orthogonal, so that each combination of all variables (such as the concentration of non-T cells) was present at all levels of every other variable (for example, the concentration of T cells and the dose of mitogen). This allowed the use of multi-way analyses of variance to assess the effects of each variable and their interactions as previously described for multivariable microcultures (Farrant *et al.*, 1980). The highest level of interaction was used for the error variance (Armitage, 1971), while replication of each individual combination of culture variables increased the number of degrees of freedom, giving a still better estimate of error variance. For brevity, analyses of variance are not shown; however, all of the positive observations described in the 'Results' and commented on in the 'Discussion' have a probability of significance of $P<0·05$. Most have a value of $P<0·001$.

Assays

The procedure and controls for the assay for the specific antibody and total immunoglobulin have been described (Brenner & Munro, 1981). Briefly, Linbro flexible plates were coated with tetanus antigen or affinity purified antiglobulins. Non-specific binding was blocked with 0·2% BSA and 0·2% gelatine in PBS, the plates washed and test supernatants added. Radio-labelled anti-IgG or IgM was then added, the counts bound measured and the antibody present determined from comparison with standard curves derived from the binding of radiolabelled antiglobulin to affinity purified IgG anti-tetanus antibody or to purified IgG or IgM.

Factor Preparation

Peripheral blood mononuclear cells (PBM) were obtained from single donors by leucophoresis. They were separated into T and non-T cells as already described. Irradiated T cells were cultured with autologous B cells at a ratio of 2:1 in 25 ml Nunc tissue culture flasks. Cells (1×10^7) grown in 5 ml of supplemented Iscove's medium—IMDM(TAS)—and 4 μg/ml of PWM were added to each flask at the beginning of culture. After 36 hr the cells were washed four times in fresh Iscove's medium and recultured for a further 72 hr. At the end of this period, the supernatants were harvested and passed down a Sepharose-4B column, to which anti-PWM antibody had been coupled. All supernatant used experimentally, contained <2 ng/ml of PWM (<300 pg/ml in final culture), as determined by a solid-phase radioimmunoassay (North & Brenner, 1983), and was passed through a 0·22 μm Millipore filter. This factor-containing supernatant was called conditioned medium (CM).

Cycloheximide

To ensure antibody and immunoglobulin detected were genuinely synthesized and did not represent carry over of immune complexes, cycloheximide was added to control cultures. This produced > 90% inhibition of both antibody and immunoglobulin production (Brenner & Munro, 1981a).

RESULTS

We have previously described a system for the production of anti-tetanus antibody from human irradiated T and B cells grown in serum-containing medium (Brenner & Munro, 1981a). The antibody produced by these cultures is almost exclusively IgG. Figure 1(a,b,c)

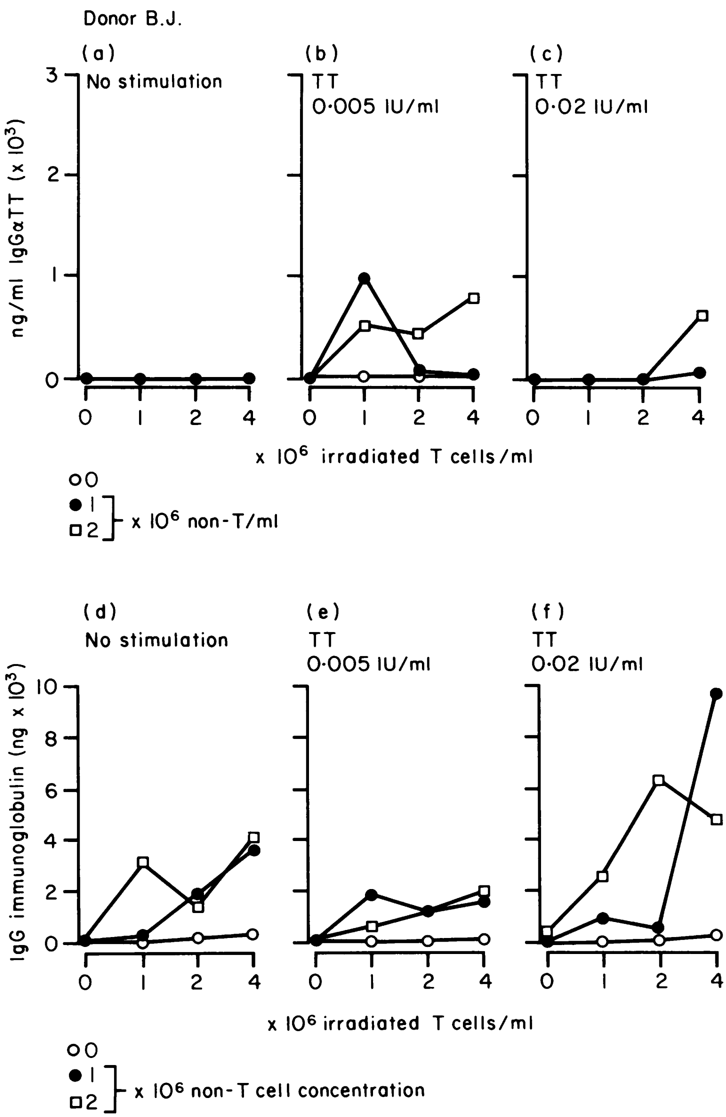

Figure 1. Panels (a), (b) and (c) show the anti-tetanus antibody production in serum-free culture in response to stimulation with two different doses of tetanus toxoid. Each line represents the antibody produced by one concentration of non-T cells after adding increasing numbers of irradiated T cells. Panels (d), (e) and (f) show the total IgG present in the same wells.

illustrates that serum-free microcultures are also able to support specific antibody production. The figure shows the effects of adding back increasing numbers of irradiated T cells to non-T cells from an *in-vitro* responder (B.J.) immunized 6 months previously. In the absence of T-cell help no antibody was produced. As the number of irradiated T cells added was increased, the initial help for antibody production was reversed, although with this donor, the inhibitory effect of too many T cells was seen only when the non-T concentration was $<2 \times 10^6$/ml. The figure also shows that the dose of antigen affects the response and

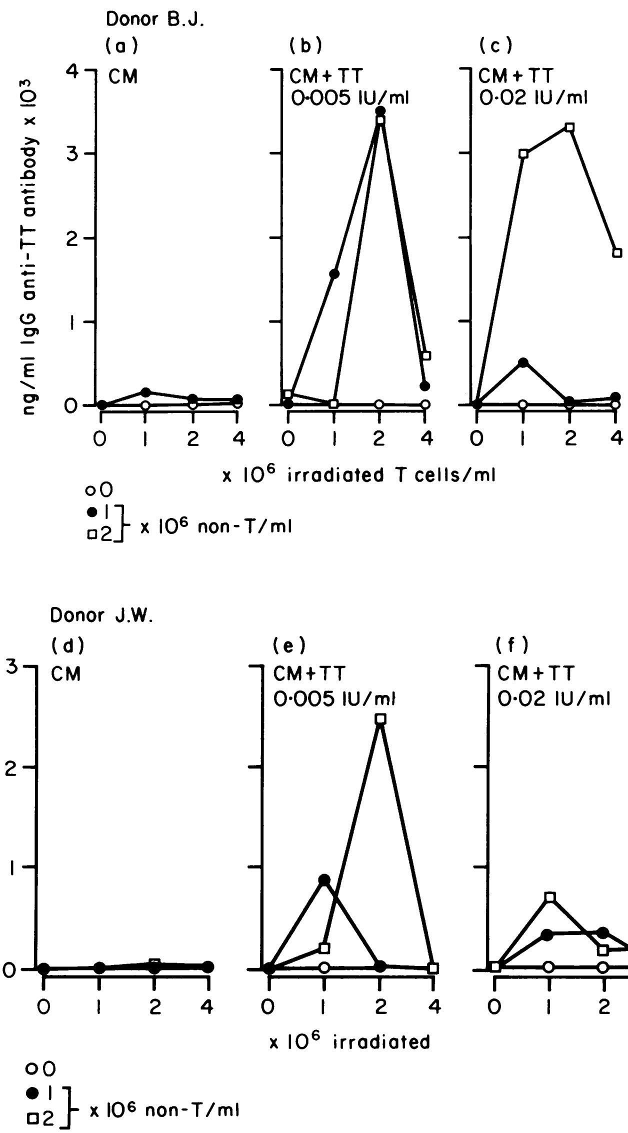

Figure 2. Panels (a), (b) and (c) show the effects of adding 15% conditioned medium, either alone or with two different doses of TT, to cells from the donor (B.J.) used in Fig. 1. Each line represents the antibody produced by one concentration of non-T cells, after adding back increasing numbers of irradiated T cells. Panels (d), (e) and (f) illustrate the antibody produced by cells from a donor (J.W.), who produces < 10 ng specific anti-TT antibody in the presence of antigen alone.

the higher level of antibody is detected with the lower of the two antigen doses.

Figure 1(d,e,f) shows that TT induces little change in total IgG in the same cultures, indicating that TT is not acting as a polyclonal activator of Ig production.

The approach illustrated above, in which an antibody response was sought at different levels of the culture variables, was applied to cells obtained from immunized, *in-vitro* non-responders. There was no conversion of non-responders to responders, so that by 3 months after immunization, only three out of 14 individuals were antigen-responsive. We therefore assessed the activity of a lectin-free supernatant derived from PWM-stimulated lymphocytes (CM).

In Fig. 2(a,b,c) the enhancing effects of this CM on antibody production, are shown on the same responder individual (B.J.) already described in Fig. 1 (a,b,c). The data shown in Fig. 1 and 2(a,b,c) are from cells obtained at the same venepuncture and are directly comparable. When both CM and TT are present, antibody synthesis was increased four-fold above the levels detected with TT alone, while the range of cell concentrations and ratios permitting antibody production were also increased. More strikingly, CM is able to convert immunized non-responders into donors who synthesize high levels of anti-tetanus antibody, provided both antigen and CM are present in the cultures. This effect is illustrated in Fig. 2(d,e,f) on one such donor (J.W.), immunized 5 months previously. Conditioned medium alone (Fig. 2d), or tetanus toxoid alone (data not shown), induce barely detectable quantities of specific antibody. In the presence of both CM and TT, up to 2500 ng/ml of anti-TT antibody are synthesized (Fig. 2e,f).

Figure 2 also shows that even in the presence of conditioned medium, the production of anti-tetanus toxoid antibody remains strictly T-dependent. The addition of CM to cultures raises the proportion of individuals able to respond to TT *in vitro* 4–24 months after immunization from 15% to nearly 60% (Table 1). Table 1 also indicates that the rise in production of specific anti-tetanus toxoid IgG antibody, when both CM and antigen are present is disproportionately greater than the rise in total IgG, so cannot be attributed to a non-specific rise in total IgG synthesis.

DISCUSSION

We have used serum-free microcultures to investigate the mechanism for the low frequency of *in-vitro* antigen-responsiveness following immunization. This

Table 1. Optimum antibody production using lectin-free conditioned medium

		CM		TT		CM+TT	
Donor	Months from last immunization*	Anti-Tet. Ab (ng/ml)†	Total IgG (μg/ml)†	Anti-Tet. Ab (ng/ml)	Total IgG (μg/ml)	Anti-Tet. Ab (ng/ml)	Total IgG (μg/ml)
N.P.‡	9	14	4·5	213	1·7	2974	11·2
S.J.‡	14	11	21·9	93	0·6	1656	36
S.S.§	7	<5	6·3	<5	<0·1	270	4·8
M.B.§	26	8	7·4	<5	0·3	622	24
T.J.§	11	<5	16·2	<5	0·15	876	41·2
C.H.§	9	<5	4·4	<5	0·2	1334	9·8

* Donors were immunized with 0·5 IU adsorbed TT (Wellcome) s.c.

† Optimum antibody and immunoglobulin concentrations are quoted as ng or μg/ml/10^6 B cells.

‡ Individuals who respond to antigen alone.

§ Individuals who fail to respond to antigen alone.

No specific antibody was produced in any donor in the absence of both CM and TT.

Results shown are the means of duplicate cultures, at cell combinations producing optimal AB. Analysis of variance (see 'Methods') indicates TT+CM induce higher quantities of antibody than either alone ($P < 0{\cdot}001$) both at any given combination of variables and overall.

technique makes it possible to determine the effects of antigen dose, cell number, and T:B cell ratio, varied both individually and together.

It is apparent from Fig. 1 that even in responder individuals, there are few conditions under which optimal antibody production occurs. The ratios of T:non-T required for optimal antibody production are dependent on both the total cell numbers of each type and the antigen dose.

In the hanging-drop system, specific antibody synthesis by responder individuals occurs at substantially lower antigen doses than in conventional culture techniques (Brenner & Munro, 1981a). Moreover, the specific antibody response is significantly reduced if higher antigen levels (>0·02 IU/ml) are used. It is difficult to attribute this observation to the induction of suppressor T cells, as the phenomenon occurs even when the T lymphocytes are irradiated before culture. B cells themselves can be the target for antigen induced suppression of antibody responses (Stevens, 1981) and a similar mechanism may be operating in these cultures. The apparent weak response to the higher dose of tetanus antigen (0·05 IU/ml) cannot simply be a result of free antigen in the culture supernatant interfering with specific antibody binding in the radio-immunoassay, for such inhibition occurs only at substantially higher concentrations of free antigens (Stevens & Saxon, 1978).

We examined whether the observed failure of *in-vitro* responsiveness to antigen by most donors 3 months after immunization could be overcome by providing the appropriate combination of culture conditions. Despite an extensive survey of culture variables, the proportion of long-term responders remained at under 20% (Table 1).

An alternative explanation for unresponsiveness *in vitro*, is that most donors fail to produce adequate quantities of the B-cell growth factors required for the development of a measurable antibody response. Two main groups of T cell-derived differentiation-inducing factors appear to act on B cells. One type bear MHC antigens, are both MHC-restricted and antigen-specific and may substitute for specific T-cell help (Marrack & Kappler 1975; Howie & Feldmann, 1977; Uytedehaag, Heijnen & Ballieux, 1978; Zanders *et al.*, 1983).

The second group are released in response to antigenic stimulus, but are not in themselves antigen-specific (Schimpl & Wecker, 1975; Swain *et al.*, 1982; Uytedehaag *et al.*, 1978; Sjöberg, Andersson & Møller, 1972). Instead, they enhance the growth and differentiation of any B cell in the appropriate state of activation. These non-specific factors do not usually carry MHC antigens, and are genetically unrestricted. In mice, there appear to be a number of interacting non-specific factors, which have as their target, B cells in different stages of activation (Schimpl & Wecker, 1975; Farrar *et al.*, 1981; Swain *et al.*, 1982). These factors may represent a positive feedback capacity within the immune system, allowing the expansion of specific, antigen-selected, B-cell clones. Thus, antigens such as tetanus toxoid induce the release of non-specific helper factors from immune T cells (Mudawwar *et al.*, 1978).

We considered that the rapid decline of *in-vitro* B-cell response to antigen with time after immunization might be due to a similar decline in the number of circulating T cells able to release B-cell non-specific differentiation factors after challenge with antigen. We therefore added back these factors in the form of a lectin-free conditioned medium, previously shown to contain factors inducing B-cell proliferation and differentiation (North & Brenner, 1983). Figures 1 and 2 indicate that differentiation factors may well be limiting, even in cultures of lymphocytes from high responder individuals, for their addition both increases the maximum amount of antibody synthesized, and broadens the range of cell concentrations and ratios under which antibody is detected. More strikingly, five donors, apparent non-responders to antigen, could be converted to high antibody producers if both antigen and CM were present (Fig. 2, Table 1). Although CM induces polyclonal activation of B cells, the anti-tetanus antibody component is seen only when both CM and antigen are present, when specific antibody rises disproportionately to the total IgG detected (Table 1). The helper factor present in CM and responsible for these effects, falls into the non-specific group. The activity is not absorbed by TT antigen or anti-DR coupled affinity columns, is genetically unrestricted, and is pH 2-resistant. Gel filtration indicates the material has a mol. wt. range of 30–50,000 (unpublished data).

In this system, antigen-induced B-cell differentiation is strictly T-dependent, even in the presence of conditioned medium (Fig. 2). In other words, although the helper factor contained in CM may be expanding specific B cells, it is not acting as 'classical' T cell-replacing factor(s) (TRF). However, even material which unequivocally contains TRF activity may not act directly on B cells when a soluble antigen like tetanus toxoid is the antigenic stimulus: murine TRF can be induced by protein antigens, but is much more effective in restoring the antibody response to particulate materials (Marrack & Kappler, 1975; Waldmann & Munro, 1974). The precise categorization of this helper factor therefore remains open.

Microculture systems that use low absolute numbers of lymphocytes have been extensively applied to limiting dilution assays of specific T- and B-cell frequency (Lefkovits & Waldmann, 1979; Smith & Callard, 1982). Although this is a potential application of the human antibody-producing system we have described, it is also a potential source for criticism. It might be argued that when so few cells are co-cultured then one or more of the cell types required for successful interaction will be limiting; the apparently restricted range of cell concentrations and ratios under which antibody is produced may be an artefact representing limiting dilution effects. There are two reasons why this explanation is unlikely. First, there is failure of antibody production at cell concentrations and ratios both lower and higher than those appropriate for antibody synthesis. Limiting dilution effects should be observed only at low cell numbers, after which the response should plateau. Second, the addition of CM increases the range of conditions under which antibody production occurs, and converts non-responders to responders. This indicates that failure of antibody synthesis is not due to a limiting number of B cells, but is a consequence of limiting quantities of differentiation factors for antigen-stimulated B cells.

It is clear from studies using antigen suicide techniques (Brenner & Munro, 1981b) and limiting dilution analysis (Smith & Callard, 1982), that the initiation of antigen-dependent antibody responses in man requires antigen-specific T cells. The results in this paper indicate that non-specific help is necessary as well, if an optimal antibody response is to develop.

REFERENCES

ARMITAGE P. (1971) *Statistical Methods in Medical Research*, Section 8.2. Blackwell Scientific, Oxford.

BRENNER M.K., BRIGHT S. & MUNRO A.J. (1981) The MHC and cell interactions in man. *Trans. Proc.* **13**, 1527.

BRENNER M.K. & MUNRO A.J. (1981a) T cell help in human in vitro antibody producing systems: role of inhibitory T cells in masking allogeneic help. *Cell. Immunol.* **5**, 280.

BRENNER M.K. & MUNRO A.J. (1981b) Human anti-tetanus antibody response in vitro: autologous and allogeneic T cells provide help by different routes. *Clin. exp. Immunol.* **46**, 171.

CALLARD R.E. (1979) Specific *in vitro* antibody response to influenza virus by human blood lymphocytes. *Nature (Lond.)*, **282**, 734.

FARRANT J., CLARK J.C., LEE H., KNIGHT S.C. & O'BRIEN J. (1980) Conditions for measuring DNA synthesis in PHA stimulated human lymphocytes in 20 μl hanging drops with various cell concentrations and periods of culture. *J. immunol. Meth.* **33**, 301.

FARRAR J.J., BENJAMIN W.R., HALFIKER M.L., HOWARD M., FARRAR W.L. & FULLER-FARRAR J. (1982) The biochemistry, biology and role of IL-2 in the induction of cytotoxic T cell and antibody forming B cell responses. *Immunol. Rev.* **63**, 129.

HOWIE S. & FELDMANN M. (1977) In vitro studies on H-2 linked unresponsiveness to synthetic polypeptides. III. Production of an antigen specific T helper cell factor, to (T,G)A-L Eur. *J. Immunol.* **27**, 417.

ISCOVE N.N., GUILBERT L.J. & WEYMAN C. (1980) Complete replacement of serum in primary cultures of erythropoietin-dependent red cell precursors (CFU-E) by albumin, transferrin, iron, unsaturated fatty acid, lecithin and cholesterol. *Exp. Cell Res.* **126**, 121.

ISCOVE N.N. & MELCHERS F. (1979) Complete replacement of serum by albumin, transferrin and soybean lipid in cultures of lipopolysaccharide-reactive B lymphocytes. *J. exp. Med.* **147**, 923.

LEFKOVITS I. & WALDMANN H. (1979) *Limiting dilution analysis of cells in the immune system*. Cambridge University Press.

MARRACK P.C. & KAPPLER J.W. (1975) Antigen specific and non-specific mediators of T cell and B cell co-operation. I. Evidence for their production by different T cells. *J. Immunol.* **114**, 1116.

MITCHELL D.M., FITZHARRIS P., KNIGHT R.A. & SCHILD G.C. (1982) Kinetics of specific in vitro antibody production following influenza immunisation. *Clin. exp. Immunol.* **48**, 491.

MUDAWWAR F.B., YUNIS E.J. & GEHA R.S. (1978) Antigen specific helper factor in man. *J. exp. Med.* **148**, 1032.

NORTH M.E. & BRENNER M.K. (1983) Induction of immunoglobulin and antigen dependent antibody synthesis in human lymphocytes using supernatants from mitogen stimulated cultures. *Immunology*, **48**, 157.

SCHIMPL A.L. & WECKER E. (1972) Replacement of T cell function by a T cell product. *Nature New Biology*, **237**, 15.

SJÖBERG O., ANDERSSON J. & MØLLER G. (1972) Reconstitution of the antibody response in vitro of T cell deprived spleen cells by supernatants from spleen cell cultures. *J. Immunol.* **109**, 1379.

SMITH C.M. & CALLARD R.E. (1982) Antigen specificity and frequency of autologous and allogeneic helper T cells in the in vitro production of antibody against influenza virus, by human blood lymphocytes. *Eur. J. Immunol.* **12**, 558.

STEVENS R.H. (1981) Immunoglobulin bearing cells are the target for the antigen induced inhibition of Pokeweed mitogen stimulated antibody production. *J. Immunol.* **127**, 968.

STEVENS R.H. & SAXON A. (1978) Immunoregulation in humans. Control of antitetanus antibody production after booster immunization. *J. Clin. Invest.* **62**, 1154.

SWAIN S.C., WETZEL G.D., SOUBRAIN P. & DUTTON R.W.

(1982) T cell replacing factor in the B cell response to antigen. *Immunol. Rev.* **63,** 111.

Uytdehaag F., Heyinen C.J., Ballieux R.E. (1978) Induction of antigen-specific human suppressor T lymphocytes in vitro. *Nature (Lond.)*, **271,** 556.

Waldmann H. & Munro A.J. (1974) T cell dependent mediator in the immune response. *Nature (Lond.)*, **243,** 356.

Zanders E.D., Fischer A., Smith S., Beverley P.C.L. & Feldmann M. (1983) Antigen specific and non-specific helper activities derived from supernatants of human influenza virus-specific T cell lines. *Immunology*, **48,** 361.